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METHODS FOR THE STANDARDIZATION OF BIOLOGICAL STAINS[1]

Part IV

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THE TRIPHENYLMETHANE DERIVATIVES

ABSTRACT.—**In this paper are given methods for determining the suitability of certain dyes of the triphenylmethane group for certification by the Commission on Standardization of Biological Stains. These methods have been developed by the Commission, in coöperation with the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture. The dyes for which the methods are given in the present paper are: Malachite green, brilliant green, light green SF yellowish, fast green FCF, basic fuchsin (rosanilin and pararosanilin), acid fuchsin, methyl violet, crystal violet, gentian violet, methyl green and anilin blue. For each of these dyes, methods are discussed under the following headings: (1) identification or qualitative examination; (2) quantitative analysis; and (3) biological tests.**

Biological stains belonging to the triphenylmethane series of dyes are examined quantitatively by means of reduction with titanous chloride solution in conjunction with a suitable buffer. The general usefulness of such a method has been discussed in Part I of this series and a detailed outline given in Part III.[1] For the convenience of the analyst, however, the general formula for the calculation of the dye content of any sample is repeated:

[1]The previous papers of this series have appeared in Stain Techn., **8**, 87, 95, 121. 1933.

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$$\frac{\text{cc. } TiCl_3 \text{ used} \times \text{normality factor} \times \text{molecular weight of } \frac{1}{2}\text{dye} \times 100}{\text{weight of sample} \times \text{no. of hydrogen equivalents} \times 1000} = \text{Per cent dye; or}$$

$$\frac{\text{cc. } TiCl_3 \text{ used} \times \text{normality factor} \times 10 \times 100}{\text{cc. of N/10 } TiCl_3 \text{ required per gram of dye} \times \text{weight of sample}} = \text{Per cent dye}$$

The method for the qualitative analysis of these stains is for the most part spectrophotometric in character, and has been described in Parts I and III of this series of papers. Any other tests will be described under the identification of the specific dye to which they are applicable.

MALACHITE GREEN, C. I. NO. 657[4]

Identification: Malachite green is the chloride or oxalate of p,p′-tetramethyldiaminotriphenylcarbinol anhydride, $C_{23}H_{25}N_2Cl$ or $2C_{23}H_{25}N_2 + 3C_2H_2O_4$. The absorption ratio of the dye (E at 605 mμ/E at 635 mμ) is 0.83. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water. To differentiate between the chloride and the oxalate, dissolve approximately 0.1 g. of the dye in 50 cc. of cold water and precipitate the dye base by the addition of a slight excess of dilute NaOH. Filter off the base and divide the filtrate into two parts. Heat one part to boiling and add dilute $Ca(OH)_2$ solution. A fine white crystalline precipitate indicates that the original dye was the oxalate. Make the other portion of the filtrate slightly acid with dilute HNO_3 and if a copious white precipitate is obtained upon addition of dilute $AgNO_3$, the original dye contained chloride.

Method of Analysis: Dissolve 0.4 g. of dye in 100 cc. of distilled water and 50 cc. of 95% alcohol, heat to boiling, add 30 cc. of a 30% sodium tartrate solution and titrate, *slowly*, with a standard solution of N/10 $TiCl_3$ until the dye solution becomes a light straw color. The following data are used in calculating the percentage of anhydrous dye in the original sample:

(1) Molecular weight, $C_{23}H_{25}N_2Cl$	364.673
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	54.844
(2) Molecular weight, $2C_{23}H_{25}N_2 + 3C_2H_2O_4$	928.480
Hydrogen equivalents per mol of dye	4.
cc. of N/10 $TiCl_3$ per gram of dye	43.081

Biological Tests: Malachite green is tested for use as a counterstain in plant histology. Botanical material (preferably plant pathological material), after fixation in a suitable fixative (e. g., Carnoy's or Farmer's fluid) and embedding in paraffin, is sectioned and stained with 0.5% fast green in 95% alcohol, distilled water, or in clove oil, either with or without previous staining in 1% aqueous safranin. The malachite green is applied for one minute when used alone, or for 20 seconds following 20 minutes of the safranin. When used alone, the stain should reveal clearly all such histological elements as the cell walls, endodermis, bast, cytoplasm, nuclei, and chloroplasts. When used following safranin on plant pathological material, the host nuclei, xylem, and cutinized walls, as well as the nuclei of the infecting fungus, should appear red. The cytoplasm and cellulose walls of the host should appear green.

[4]This is the number under which the dye is listed in the "Colour Index," published by the Society of Dyers and Colourists, Bradford, Yorkshire, 1924.

BRILLIANT GREEN, C. I. No. 662

Identification: Brilliant green is the acid sulphate of p, p′-tetraethyldiaminotriphenylcarbinol anhydride, $C_{27}H_{33}N_2SO_4H$. The absorption ratio of the dye (E at 620 mμ/E at 650 mμ) is 1.51. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 75 cc. of distilled water and 75 cc. of 95% alcohol, add 10 g. of sodium acid tartrate, heat to boiling and titrate to a *permanent*[5] end point. At the end point the solution changes in color from light green to pale yellow. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	482.352
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	41.463

Biological Tests: Brilliant green is tested as to its suitability for determining the presence of the colon organism in drinking water. The object of the dye is to prevent formation of gas by the bacillus of gas gangrene, *Bacillus Welchii*. For this purpose standard methods prescribe a medium containing 2% dried oxgall with a 1/75,000 dilution of brilliant green after addition to the water to be tested. In testing a dye sample, varying amounts of brilliant green are added to the bile medium for one series of tests, using a sufficient variety of solutions to be certain of growth in the most dilute and absence of growth in the most concentrated. A second series of tests is set up with varying dilutions of pure cultures of the colon organism (*Bacterium coli*) on the standard medium as above mentioned, while another comparison is made in standard lactose broth. A satisfactory sample should allow 10% gas production in 1 to 3 days by the colon organism, but not by *Bacillus Welchii* at the dilution called for in the standard medium. The object of the long series of tests is to check up on the delicacy of the medium as used, and on the effect of varying the quantities of brilliant green.

FAST GREEN FCF[6]

Identification: Fast green FCF is the disodium salt of p, p′-dibenzyldiethyldiamino-p″-hydroxytriphenylcarbinol trisulfonic acid anhydride, $C_{37}H_{34}O_{10}N_2S_3Na_2$. The absorption ratio of the dye (E at 605 mμ/E at 635 mμ) is 0.76. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Add 30 g. of sodium acid tartrate to 300 cc. of water, heat until dissolved and cool slightly with CO_2. Then add 1 g. of dye dissolved in 100 cc. of water and titrate with standard $TiCl_3$ solution. The temperature of the solution should be kept at or near 70° C. during the titration. The end point varies in color from yellow to reddish brown. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	808.474
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	24.738

Biological Tests: Fast green is tested for use as a cytological counterstain. Sections of root tips or buds are fixed in Flemming's fluid and are stained in 1% aqueous safranin for from 15 minutes to 2 hours, rinsed in water and differentiated with 0.2% fast green

[5]There is a strong tendency for the color to return while titrating this dye, which is more pronounced in aqueous than in alcoholic solutions.

[6]This dye is not listed in the Colour Index.

in 95% alcohol until the chromatin and nucleoli remain red. The slides are then passed thru absolute alcohol to xylol and mounted in balsam. Chromosomes and other chromatin bodies as well as nucleoli and lignified walls should appear bright red, while the spindles, cellulose walls, and cytoplasm are green.

LIGHT GREEN SF YELLOWISH, C. I. No. 670

Identification: Light green SF yellowish is the disodium salt of p,p′-dibenzyldiethyldiaminotriphenylcarbinol trisulfonic acid anhydride, $C_{37}H_{34}O_9N_2S_3Na_2$. The absorption ratio of the dye (E at 620 mμ/E at 650 mμ) is 0.98. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Add 30 g. of sodium acid tartrate to 300 cc. of water, heat until completely dissolved and cool slightly with CO_2. Then add about 1 g. of dye dissolved in 100 cc. of water and titrate with N/10 $TiCl_3$ solution. The temperature of the solution should be 70° C. during the titration. The end point is a sharp change from light green to yellow. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	792.474
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	25.238

Biological Tests: Light green is tested by the same procedure as fast green.

BASIC FUCHSIN, C. I. No. 677

Identification: Stains marketed under the name of basic fuchsin may be either the chloride or acetate of pure[7] rosanilin, pure pararosanilin, or mixtures of these two dyes. Rosanilin (the base) is triaminodiphenyltolylcarbinol. Its chloride has the formula, $C_{20}H_{20}N_3Cl$, its acetate is $C_{20}H_{20}N_3 \cdot C_2H_3O_2$. Pararosanilin (the base) is triaminotriphenylcarbinol. Its chloride has the formula, $C_{19}H_{18}N_3Cl$, and its acetate is $C_{19}H_{18}N_3 \cdot C_2H_3O_2$. The absorption ratio of rosanilin chloride or acetate (E at 530 mμ/E at 560 mμ) is 0.89. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water. Under the same conditions the value of the ratio for pararosanilin chloride or acetate is 1.13. The proportionate amounts of rosanilin and pararosanilin in mixtures of the two may be estimated from the variation of the ratio of the sample in question from the ratios of the pure dyes as given. For example, a 50-50 mixture of the two dyes would have an absorption ratio of approximately 1.01 (halfway between the values 0.89 and 1.13). To test for acetate, add 1 cc. of 6 N. H_2SO_4 to 0.2 g. of the dye dissolved in 5 cc. of distilled water, and heat. The odor of acetic acid escaping from the hot mixture indicates the presence of acetate in the original sample.

Method of Analysis: Dissolve 0.3 g. of dye in 75 cc. of 95% alcohol and 50 cc. of distilled water, heat to boiling, add 25 cc. of 30% sodium tartrate solution, and titrate with standard N/10 $TiCl_3$ solution to a yellow end point. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Rosanilin:	*Chloride*	*Acetate*
Molecular weight	337.641	361.208
Hydrogen equivalents per mol of dye	2.	2.
cc. of N/10 $TiCl_3$ per gram of dye	59.234	55.370

[7] "Pure" is used here in the sense of freedom from homologous dyes, and not in the sense of freedom from such impurities as inorganic salts, colorless organic substances, and sometimes subsidiary dyes.

Pararosanilin:	*Chloride*	*Acetate*
Molecular weight	323.625	347.192
Hydrogen equivalents per mol of dye	2.	2.
cc. of N/10 $TiCl_3$ per gram of dye	61.800	57.605

Biological Tests: Basic fuchsin is tested for two different purposes. (1) For use as a stain for the tubercle organism, and (2) for use in the Endo medium for distinguishing between the coli and aerogenes types of bacteria.

(1) In testing as a stain for the tubercle organism, smears are covered with carbol fuchsin (1 part of 3% alcoholic fuchsin to 9 parts of 5% aqueous phenol) and heated on a water bath for at least 3 minutes. They are then rinsed in water and differentiated in 70% alcohol containing 1% hydrochloric acid until practically no red color remains visible to the naked eye. They are again rinsed in water; treated with 95% alcohol for 30 seconds; rinsed once more and counterstained with dilute (i. e., about 0.1%) aqueous methylene blue. The slides are then rinsed and dried and examined under the microscope. The tubercle organisms should be distinctly red, while other bacteria, leucocytes and debris appear blue.

(2) In testing for use in the Endo medium, 1 cc. of a 1% solution in 95% alcohol of the sample to be tested is mixed with 0.125 g. of anhydrous sodium sulfite dissolved in one or two cubic centimeters of distilled water. The solution should then be a faint pink or straw color, or sometimes light brown, but without noticeable precipitate. This decolorized solution is added to 100 cc. of melted lactose agar of the standard formula. The color of this medium should then be a light pink, which fades almost entirely upon cooling. Before cooling it is poured into a Petri dish and allowed to harden. The surface is then streaked in parallel lines with *Bacterium coli* and *Bacterium aerogenes* and allowed to incubate for 24 hours. A good sample should show red growth with a strong metallic sheen for the former organism, pink growth without metallic sheen for the latter, and no reddening of the medium except where growth has occurred.

GENTIAN VIOLET

Dyes furnished under this label are frequently poorly defined mixtures of methylated rosanilins, of the type usually designated as methyl violet. In some cases crystal violet is marketed under the name gentian violet, and at times products of this nature have both names on the label. Since crystal violet is more satisfactory for bacteriological work and for those histological purposes where a deep blue-violet is required, and methyl violet 2B more desirable in histological procedures where a reddish-violet is called for, users should specify which of the two is desired and should avoid entirely the term gentian violet. It is hoped that when users have been sufficiently educated, distributors of stains can be induced to label these violets methyl or crystal, as the case may be, and to abandon the meaningless name gentian violet.[8]

When a dye is submitted under the name gentian violet, the nature of the dye which predominates is determined spectrophotometrically, and the analysis and biological tests are made as described under that dye. (See methyl violet, C. I. No. 680, and crystal violet, C. I. No. 681.)

METHYL VIOLET 2B, C. I. No. 680

Identification: Methyl violet 2B is a mixture of the more highly methylated fuchsins, principally pentamethylpararosanilin chloride, that is, the chloride of pentamethyl-

[8] For a more complete discussion of these violet stains, see H. J. Conn, Biological Stains, (*2nd Ed.*), 1929, p. 101.

triaminotriphenylcarbinol anhydride. For purposes of estimation the formula $C_{24}H_{28}N_3Cl$ is employed. The absorption ratio of this dye (E at 575 mμ/E at 605 mμ) is 1.41. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 75 cc. of 95% alcohol and 75 cc. of distilled water, heat to boiling, add 30 cc. of 30% sodium tartrate solution, and titrate slowly with N/10 $TiCl_3$ solution. The end point is sharp, changing from pink to pale reddish-brown. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	393.705
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	50.800

Biological Tests: Methyl violet 2B is tested by the same procedure as crystal violet, C. I. No. 681. Results should be the same except that the violet color obtained is of a redder shade.

CRYSTAL VIOLET, C. I. No. 681

Identification: Crystal violet is the chloride of hexamethylpararosanilin, that is, hexamethyltriaminotriphenylcarbinol anhydride, $C_{25}H_{30}N_3Cl$. The absorption ratio of this dye (E at 575 mμ/E at 605 mμ) is 1.06. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 60 cc. of distilled water and 100 cc. of 95% alcohol. Heat to boiling, add 30 cc. of 30% sodium tartrate solution, and titrate with N/10 $TiCl_3$ solution. As the reaction is very slow, especially near the end point, the titration must be carried out slowly. The reduction products have a strong tendency to precipitate during the course of the titration. This precipitation may be prevented or, in any case, lessened by keeping the solution hot thruout the titration and by adding more alcohol to the solution. The end point is a sharp change from pink to pale yellow. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	407.721
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	49.053

Biological Tests: Crystal violet is tested in three different procedures: (1) the Gram stain; (2) the Flemming triple stain; (3) followed by iodine in the staining of cytological preparations.

(1) In testing crystal violet for staining bacteria by the Gram technic, the sample is made up in a 0.5% aqueous solution, without the use of anilin oil or any other mordant, and bacterial smears are stained by the following procedure: Crystal or gentian violet 1 minute, Lugol's iodine solution 1 minute, 95% alcohol 30 seconds, 0.05% safranin solution 10 seconds. In making the test, a weakly Gram-positive organism and a Gram-negative organism are tested; the former should appear blue, the latter red.

(2) In testing the sample in the Flemming stain, the following procedure is followed: Root tips or buds, fixed in Flemming's fluid, are embedded in paraffin and sectioned. Sections are stained for 20 minutes to 2 hours in 1% aqueous safranin, rinsed in water (sometimes differentiated in weakly acidulated alcohol and then rinsed in water), stained from 2 to 10 minutes in a 1% solution of the sample being tested, again rinsed, dehydrated in 95% and absolute alcohol, differentiated in a saturated solution of orange G in clove oil, cleared in xylol and mounted in Canada balsam. A good stain

should show red chromosomes and nucleoli, while metabolic chromatin should stain a deep purple on an orange cytoplasm.

(3) In the test with iodine, paraffin sections of root tips or buds (fixed in Navashin's fluid or one of its modifications) are stained for 3 to 6 minutes in a 1% aqueous solution of the dye, rinsed in water, and treated for 15 to 50 seconds in equal parts of 1% iodine in 80% alcohol and 1% potassium iodide in 80% alcohol. They are next rinsed in absolute alcohol and differentiated in clove oil. By this technic, chromatin and nucleoli should stand out a deep violet on a colorless protoplasm.

METHYL GREEN, C. I. No. 685

Identification: Methyl green is the zinc chloride double salt of ethylhexamethylpararosanilin bromide, $C_{27}H_{35}N_3ClBr + ZnCl_2$. The absorption ratio of this dye (E at 620 mμ/E at 650 mμ) is 0.90. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 75 cc. of distilled water and 75 cc. of 95% alcohol, heat to boiling, add 25 cc. of 30% sodium tartrate solution, and titrate with N/10 $TiCl_3$ solution. Several color changes occur, but the final end point is practically colorless and unmistakable. The following data are used to calculate the percentage of anhydrous dye in the original sample:

Molecular weight	652.971
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	30.629

Biological Tests: Methyl green is tested in staining gonorrhoeal smears by the Pappenheim-Saathof stain, using the formula given on page 119 of Biological Stains, 2nd edition. With a good sample the cocci should be bright red and the nuclei green (not purplish).

ACID FUCHSIN, C. I. No. 692

Identification: Acid fuchsin should be the disodium salt of the trisulfonic acid of rosanilin or pararosanilin or mixtures of these two. Some samples may contain the mono or disodium salts of the disulfonic acid or the trisodium salt of the trisulfonic acid, but if such is the case they are not nearly as satisfactory as those which contain only the disodium trisulfonate. Samples of acid fuchsin are considered for analytical purposes as a mixture of equal parts of trisulfonated rosanilin and pararosanilin, unless specific information concerning the character of the fuchsin used for the starting material has been obtained from the dye manufacturer. Considerable diversity in the values of the absorption ratio (E at 530 mμ/E at 560 mμ) has been found with this stain. Values which fall within the limits of 0.75 and 0.95 are characteristic of the dye. The solvent employed is 95% alcohol.

Method of Analysis: Dissolve 0.5 g. of dye in 150 cc. of distilled water and 50 cc. of 95% alcohol,[9] add 10 g. sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution. The end point is a distinct change from pale red to yellow. The following data are used for determining the percentage of anhydrous dye in the original sample when calculated as:

(1) The disodium salt of rosanilin trisulfonic acid:

Molecular weight, $C_{20}H_{17}N_3O_9S_3Na_2$	585.346
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	34.168

[9]The alcohol may be omitted with many of the better quality samples.

(2) The disodium salt of pararosanil n trisulfonic acid:

Molecular weight, $C_{19}H_{15}N_3O_9S_3Na_2$	571.330
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	35.007

(3) A mixture of equal parts of (1) and (2):

Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	34.588

Biological Tests: Acid fuchsin is tested in the Van Gieson connective tissue stain on animal tissue fixed in formalin or other suitable fixing fluid. The procedure followed is given in Biological Stains, 2nd edition, page 40. The tissue elements stained by the acid fuchsin should show a clear red, contrasting well with hematoxylin and picric acid. A good sample does not show great tendency to fade.

Acid fuchsin is also tested in the anilin blue connective tissue stain by the procedure given in Biological Stains, 2nd edition, page 114. The same criteria are used in judging a good sample with this technic as with the Van Gieson stain.

Andrade Indicator: Acid fuchsin is tested as to its behavior in the Andrade indicator as follows: A 0.2% solution of the dye in 100 cc. of water is decolorized by adding normal NaOH a little at a time. Complete decolorization should be brought about without the use of more than 25 cc. of the sodium hydroxide. (The quantity of NaOH required should be stated on the label.)

ANILIN BLUE, WATER SOLUBLE, C. I. No. 707

Identification: Anilin blue consists of mixtures of the various sulfonation products of variable mixtures of phenylated rosanilin and pararosanilin, usually the latter. The average absorption ratio of the dye (E at 590 mμ/E at 620 mμ) is 1.08. Values obtained from different samples of anilin blue range from 1.03 to 1.12. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.5 g. of dye in 150 cc. of distilled water, add 15 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution. The end point will vary, depending on the sample, from yellow to dark yellow-green, and is generally easy to detect. Because of the uncertain composition of anilin blue, no attempt is made to determine the dye content. Specifications have been made which place the minimum $TiCl_3$ consumption per gram of dye at 15.64 cc. of 0.1 N. solution.

Biological Tests: Anilin blue is tested in Mallory's connective tissue stain on animal tissue fixed in Zenker's fluid and embedded in celloidin or paraffin. The procedure followed is the one given on page 114 of Biological Stains, 2nd edition. With a good sample, the collagen fibrils, reticulum, amyloid, and mucus stain blue.

ACID VERSUS NEUTRAL FORMALIN SOLUTION AS A NEUROLOGICAL FIXATIVE

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ABSTRACT.—**The fixing action of 10% formalin solution alone and with formic, acetic, propionic, butyric, lactic, monochloracetic, dichloracetic, or trichloracetic acid was studied by means of stains with silver, osmic acid and cresyl violet. The following conclusions were reached:**

1. In general, better fixation and staining was obtained with acid than without.

2. Less difference was seen in comparing one acid with another than was expected before the experiments were made.

3. Propionic, butyric, and dichloracetic showed no promise of having practical value.

4. Formic and monochloracetic acids as modifiers gave superior stains with osmic acid, while silver and cresyl violet stains of the same material were about equal to those made from formalin-acetic fixed material.

5. Lactic acid caused somewhat more distortion of tissue elements than the others but was compatible with good staining.

6. Acetic acid was most effective in concentrations of 3 to 5% while the stronger acids such as formic, monochloracetic, lactic and trichloracetic were effective in concentrations of 0.5 to 1%.

The series of experiments comprised about 80 samples of tissue consisting of central nervous system and peripheral nerves of cat. The primary object was to determine whether fixation by neutral or acidified formalin was best suited for the enumeration of nerve cells and fibers in the same piece of tissue. As a secondary objective the general character of fixation was observed both in the gross and microscopic appearance of the specimens.

Previous work (Droegmueller;[2] Davenport and Ranson)[3] had indicated that 10% aqueous formalin plus 3 to 5% acetic acid was preferable to neutral formalin for peripheral nerves when the prepara-

[1]Contribution No. 197.

[2]Droegemueller, W. H. Sensory fibers in the spinal nerves of man. Anat. Rec., **52**, Abs. 53. 1932.

[3]Davenport, H. A. and Ranson, S. W. Ratios of cells to fibers and of myelinated to unmyelinated fibers in spinal nerve roots. Am. J. Anat., **49**, 193. 1931.

tion was to be stained with both silver and osmic acid by using adjacent ends of the same nerve. The present work was done to determine whether other members of the aliphatic acids might be better than acetic.

The plan was to fix nerves or pieces of tissue containing nervous elements in a series of fixatives consisting of neutral formalin, and formalin modified by the following acids: formic, acetic, propionic, butyric, lactic, monochloracetic, dichloracetic, trichloracetic, and nitric. (The nitric was used as a sort of control on acidity itself.) After 24 hours fixation and 6 hours washing, the specimens were cut in two and one piece stained in block with osmic acid while the other was embedded unstained in paraffin. After embedding and cutting of the unstained end, the sections were stained with silver (section method)[4] and, where ganglion cells were present, separate stains (not counterstains) were made with cresyl violet.

Since the results were not as variable as originally expected, comment is made only on differences which seemed consistent in material from different cats. Myelin sheaths which had a neurilemma were stained by osmic acid after fixation in all acid mixtures as well as neutral formalin. Removal of excess formalin by washing after fixation was necessary to insure clear backgrounds. In the optic nerve, however, fixation prior to staining was much less satisfactory than direct fixation in osmic acid.

The similarity of all preparations stained by silver made a choice of acids difficult. All stains after acidified formalin were as good and many were better than after neutral formalin. The swelling of axis cylinders and the discreteness of unmyelinated fibers showed the chief variation.

The fragmentation of nerves, cut in cross section, that is almost universally seen after neutral formalin fixation, was nearly eliminated by acidification of the fixative. This fragmentation, sometimes called "paraffin artefact" appears to be a fixation artefact as well.

Formic acid was tried in concentrations of 0.25, 1, and 2% added to 10% formalin. The two higher concentrations caused the endoneurium to fix as a homogenous mass. In the silver stain large axis cylinders appeared to be somewhat swollen yet unmyelinated fibers were fairly discrete and appeared to be completely stained (tibial nerve). In osmic acid, the preservation of the sheaths was good in large fibers but small ones were often crenated. Connective tissue was very pale in both stains.

[4]Davenport, H. A. Staining nerve fibers in mounted sections with alcoholic silver nitrate solution. Arch. Neurol. & Psychiat., **24**, 690. 1930.

Acetic acid was tried in 0.5, 2, and 5%. The highest concentration was most effective in accentuating the staining of fine fibers. Large fibers and myelin sheaths were usually distorted by crenation of the fibers and vacuolation of the sheath.

Propionic, butyric, and lactic acids were used in 3.5, 4, and 4.5% concentrations respectively (about 0.5 M). The effect of propionic and butyric were similar to acetic except that they did not seem to penetrate as well. The centers of tissue blocks suffered from the same sort of distortions as with acetic acid but the distortions were more marked. Lactic acid gave a fixation similar to formic. Somewhat more swelling of large axis cylinders appeared but the roundness and integrity of the myelin sheath was retained. Unmyelinated fibers were discrete and well stained.

In the modification of formalin by the chlorinated acetic acids it was found that monachloracetic resembled acetic while dichlor- and trichloracetic acids were alike but differed from the other two members of the acetic series. Monochloracetic acid caused a great deal of swelling especially of connective tissue whereas dichlor- and trichloracetic preserved the gross shape of the tissue as well or better than neutral formalin. Acid concentrations of 1 and 2% were tried with no apparent choice. Monochlor- and trichloracids (0.25% concentration) showed somewhat better preservation of cytoplasm than fixation with plain formalin. All concentrations of monochloracetic acid in formalin gave better stains with both silver and osmic acid than formalin alone. The dichlor- and trichloracids as modifiers were better in the lower concentrations and altho osmic acid stains were usually good, silver was erratic.

As a check on acidity *per se*, 0.01 N nitric acid (final concentration) was added to 10% formalin and a specimen of tibial nerve fixed in it. Myelin sheaths and axis cylinders were distorted in that the sheaths were thinned and expanded outward against the neurilemma while the axis cylinders were frequently crenated. The unmyelinated fibers stained rather pale but were not badly clumped.

If the acids be grouped according to the histologic picture they produced (as modifiers of formalin) formic, monochloracetic, and lactic would fall together. Acetic, propionic, and butyric would form another group, dichlor- and trichloracetic another, while nitric would stand alone. The classifications might be justified theoretically in that the first three are moderately strong acids of similar molecular configuration, the second three are weaker acids, and none of the six are very active protein precipitants. Dichlor- and trichloracetic acids are molecularly similar to the others but are much stronger organic acids and are both active protein pre-

cipitants even at room temperature. Nitric is hardly comparable as it is a strong mineral acid of different molecular form.

The lessons which can be drawn from the experiments seem to be as follows:

1. The prejudice against the use of formic acid (or even its presence in formalin) is not supported by any obvious defects in neurological material subjected to silver, osmic acid, and a dye stain. In fact, formic acid seemed to be one of the best of the acid modifiers.

2. The presence of relatively minute quantities of lactic acid generated during post mortem changes is not the cause of defective fixation or staining, since lactic acid was a very good modifier for formalin.

3. Propionic and butyric acids as well as dichloracetic acid appear to have no immediate application. The first two seem to penetrate poorly, while the latter can be substituted by trichloracetic acid to advantage both as a fixative and availability of the pure acid.

4. Acetic acid will probably continue to be the most popular acid modifier but the use of formic, monochloracetic, and lactic deserves consideration.

A VARIATION OF THE PAL WEIGERT METHOD FOR STAINING MYELIN SHEATHS

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Abstract.—**A variation of the Pal-Weigert method for staining myelin sheaths is described, which is successful on some sections on which Pal's modification of the Weigert method fails; which does not require old ripened hematoxylin; and which can be completed in from three to five hours. It varies from the Pal-Weigert method by the insertion of a bath of ferric ammonium sulfate both before and after the staining with hematoxylin.**

It has been our experience in staining brain-stem sections for myelin sheaths that the Pal-Weigert method (as described by Lee, 1924)[1] is usually satisfactory when the material has been properly fixed and hardened. When certain material, however, failed to stain with the Pal-Weigert method, variations were attempted until the following method was evolved:

1. Sections of the desired thickness are mordanted in a 4% aqueous solution of ferric ammonium sulfate ($FeNH_4(SO_4)_2 \cdot 12H_2O$) (iron alum) for from 2 to 24 hours, as is convenient.
2. They are then washed for a short time in tap water.
3. Next they are stained for 1 to 2 hours in hematoxylin similar to that used for the Weigert method, made up by adding 9 volumes of solution A to one volume of solution B. (Solution A is made by adding 7 cc. of a saturated aqueous solution of lithium carbonate to 93 cc. of distilled water. Solution B is made by dissolving one gram of hematoxylin in 10 cc. of absolute alcohol.)
4. The sections are next washed for 2 or 3 minutes in tap water.
5. They are then partially decolorized in 4% ferric ammonium sulfate solution. The time of this step varies with the amount of staining and should be carried on until the gray and white matter are barely distinguishable.
6. The sections are then washed in tap water for 2 or 3 minutes.
7. Next the sections are placed in a 0.25% solution of potassium permanganate, in which the gray and white matter become clearly

[1] Lee, A. B. The Microtomist's Vade-Mecum. Blakiston's, Philadelphia. 8th ed., 1924. Sec. 857.

distinguishable when the sections are held up to the light. They are colored brown by the permanganate.

8. They are then quickly rinsed in tap water.

9. Decolorization is continued in a freshly prepared mixture of equal parts of a 1% solution of oxalic acid, and a 1% solution of sodium (or potassium) sulfite. In this solution the gray matter is bleached until it is clear and colorless (except where it contains some myelinated fibers). Steps 7 thru 9 may be repeated if needed.

10. The sections are again washed in tap water for 2 or 3 minutes.

11. They are then placed in a saturated (or weaker) solution of lithium carbonate for 5 minutes or more, where they regain the blueness which may have been lost in the decolorizing process.

12. They are again washed thoroly in tap water, after which they may be counterstained if desired, then dehydrated, cleared and mounted.

When the material has been properly fixed, the individual myelin sheaths in sections so treated are clearly and sharply stained. No difficulty is encountered in tracing the myelinated fibers along pial blood vessels of such sections. The color of the myelin sheaths and, therefore, of the white matter is a dark blue in which no red is detected, while the classical Pal-Weigert method usually results in a shade of violet, or at least a blue in which there is a tinge of red.

While this stain has been used mostly on celloidin sections cut from formalin-fixed brains which have been mordanted in a bichromate solution, it has also been successfully used on celloidin sections of formalin-fixed material which was not mordanted with bichromate, and on frozen sections of material fixed in formalin or in 10% formalin containing 2% ammonium bromide.

In the use of this stain for a large series of brain stem sections a basket of copper wire screen divided into four compartments was used to carry four sections at a time thru the solutions. By covering the copper screen with a thin coating of paraffin the solutions and sections remained unharmed by the copper. Constant shaking of the basket in the decolorizing solutions and water baths both aids and speeds up the process.

In contrast to other methods for staining myelin, the hematoxylin solution in the present method does not have to be ripened with age. Hematoxylin solutions varying in age from less than one day to eight months have been used successfully. Several sections may be stained simultaneously in the same dish, provided they are kept in order by placing them between filter papers which have been previously soaked in the stain. The hematoxylin can be used for a number of sections, even tho a precipitate forms in it. The decolorizing solutions and

the lithium carbonate solution used in the process should be replaced when they begin to become discolored.

This method differs in detail from the iron hematoxylin method described by Morgan (1926)[2] tho it is based on similar principles and requires about the same time. Morgan, however, recommends the use of 'ripened' hematoxylin. It is not as rapid as the method of Weil (1928)[3] but is more economical of stain and easier to use when a great many sections have to be stained.

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[2]Morgan, L. O. Iron hematoxylin as a myelin sheath stain and neutral red ripened by colon bacillus as a nerve-cell stain. Anat. Rec., **32**, 283. 1926.

[3]Weil, A. A rapid method for the staining of myelin sheaths. Arch. Neurol. and Psych., **20**, 392. 1928.

DESTAINING AGENTS FOR IRON ALUM HEMATOXYLIN

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ABSTRACT.—**Detailed directions for using various destaining agents after iron alum hematoxylin are given. A saturated aqueous solution of picric acid is recommended for bringing out nuclei in whole mounts of protozoa. Differentiation with a mixture of 1 part 30% hydrogen peroxide, and 2 parts, 95% alcohol, is useful for cytoplasmic structures. Greater reliability and precision are claimed for these methods as compared with the conventional differentiation in iron alum.**

Iron alum hematoxylin, altho a splendid stain both for nucleus and for cytoplasm, offers a number of difficulties when destaining is done with the usual iron alum solution:

1. Material tends toward a murky tan-color before the desired degree of decolorization is reached.
2. It is difficult to stain nuclei clearly in other than thin sections.
3. It is difficult to stain cytoplasmic structures well without overstaining the nucleus or the background. The latter is especially important when coverslips are albuminized in order to retain protozoa.
4. Slides must be washed for a long time after staining to avoid fading.

Iron alum in solution is both an acid and an oxidizer. Its destaining action is twofold: as an acid it extracts stain faster from the cytoplasm than from the nucleus; while as an oxidizer it bleaches the stain uniformly. By the use of an acid alone staining for nuclei can be favored more than with iron alum; conversely, with an oxidizer alone staining of cytoplasm is favored. Destaining in iron alum, with its concomitant ugly precipitate of basic ferric compounds, is unnecessary. Most of the defects enumerated above can be minimized or entirely eliminated by using other means of destaining. There is little that is new in technic as outlined in the paragraphs which follow. The author's aim is to render more widely known certain methods which protozoologists have found especially useful.[1]

Nuclei. Following the method advocated by Tuan (1930), the author finds picric acid invaluable in staining nuclei in whole mounts of protozoa. A satisfactory schedule for small flagellates is as follows:

[1]The author is indebted to Drs. B. P. Young and L. W. Sharp for their helpful criticisms and kindness in checking over the preparations.

1. Mordant in 4% iron ammonia alum for one hour.
2. Wash in running water or changes of distilled water for 15 minutes.
3. Stain in 0.5% hematoxylin for one hour.
4. Wash in water until no more stain comes out.
5. Destain in a saturated aqueous solution of picric acid.
6. Wash as in step 4, until no more picric acid comes out.
7. Blue by adding a drop or two of ammonia to the 70% or 80% alcohol used for dehydration.

T. T. Chen has found a 24 to 48 hour schedule, mordanting and staining equal times, very successful for studying the chromosomes of opalinids mounted in toto (personal communication).

Cytoplasm. Hydrogen peroxide is an efficient oxidizer. Kidder (1933) notes that a 10% solution of hydrogen peroxide is of value in bringing out the neuromotor system of certain ciliates. As commercial peroxide is only a 3% solution and operates rather slowly, the author has found that destaining for cytoplasm can be hastened and rendered more uniform with the following slightly different procedure:

1. Mordant and stain as above,—30 minutes mordanting and 30 minutes in hematoxylin.
2. Run up to 50% alcohol.
3. Destain in the following mixture freshly prepared at the time of using:

95% alcohol . . . . . 2 parts
Merck's "Superoxol" (a 30% peroxide) . . . 1 part

4. Rinse in one change of 70% alcohol for 10 minutes.
5. Finish dehydration and mount.

The slides remain bright blue during destaining. Differentiation can therefore be very accurately controlled.

By combining oxidizer and acid in one mixture a fairly close approximation to iron alum destaining can be obtained from the point of view of relative staining of nucleus and cytoplasm. Such a mixture is:

0.25% solution of HCl in 95% alcohol . . . . . 2 parts
"Superoxol" . . . . . 1 part

This solution, too, should be freshly made. After decolorization the slides should be washed with 70% alcohol, and blued as after picric acid. It is well to test the stronger alcohols occasionally with litmus or phenolphthalein to make sure the acid is completely neutralized.

Dobell's (1914) view that "..many 'good' Heidenhain preparations showing sharp black and white effects are in reality very bad; for the pretty pictures produced are the results of a coat of paint which masks the underlying structure" has served as a guide in this work. The solutions containing peroxide have been made up with alcohol to insure rapid penetration and consequent even staining. This precaution appears to be unnecessary with picric acid. It has not been necessary to carry out mordanting and staining in alcoholic solutions as some protozoologists have done (Dobell, 1914, Kofoid and Swezy, 1915, and Doflein, 1919).

Care must be taken that the mounting medium and xylol are at least neutral, for the stain may be as delicate as an anilin coloration, and any fading would soon become conspicuous. Demar balsam, purified according to the method given on page 141 of McClung's handbook, has given very good results. Slides of termite flagellates, destained by the peroxide method, and mounted after the briefest rinse, have retained their original bright blue color after seven months.

The hematoxylins used were from various sources including a certified American brand and a sample of Grübler's. All gave similar results.

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CAN THE ENDO MEDIUM BE STANDARDIZED?[1]

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Abstract.—**An investigation of various formulae for the Endo medium has been made, using about 20 different samples of basic fuchsin. It has been found that some samples are unsatisfactory in the standard formula of the American Public Health Association and others unsatisfactory in the formula given in the Manual of Methods for Pure Culture Study of Bacteria (of the Society of American Bacteriologists). A slight modification of the A. P. H. A. standard formula, however, is enough to make it satisfactory for any sample of basic fuchsin with which it has so far been tried. This modification is the use of 1 ml. of a 1% basic fuchsin solution instead of 0.5 ml. of a 10% solution. This modification in the formula brings about a change of the ratio of sulfite to fuchsin in the finished medium of 12½:1 instead of roughly 3:1; and it is shown that this ratio permits the use of a greater variety of different fuchsins than the one which is in the standard formula.**

One of the most puzzling problems that has faced the Commission on Standardization of Biological Stains is the standardization of basic fuchsin for the Endo medium. Some samples have proved satisfactory in the standard formula, while others have not, and there has been very little correlation of their behavior in the chemical relation of the dye.

One thing that has added to the difficulties in this problem has been the failure to understand the Endo reaction completely. It has generally been regarded as similar to the Schiff reaction for aldehyde, but certain discrepancies have been noticed that have made this explanation difficult to accept. A recent paper by Margolena and Hansen (1933) reports work carried on in coöperation with the Stain Commission investigating the theory of Neuberg and Nord (1919), that the sulfite present in the Endo medium acts as a trapping agent for small quantities of aldehyde produced by the bacteria and hence allows the medium to serve as a reagent for the detection of aldehyde production, even in instances where aldehyde does not normally accumulate. Margolena and Hansen have furnished

[1]Paper presented at the 1933 (Philadelphia) meeting of the Society of American Bacteriologists. (*Abstr. in* J. Bact., **27**, 33. 1934.) Approved Feb. 21, 1934, as Journal Paper No. 35, by the Director of the New York State Agricultural Experiment Station.

additional proof of this theory, and altho their work did not directly explain the faults of certain fuchsins in the Endo technic, it has been of considerable assistance to the present writers in solving the basic fuchsin problem.

Ever since the beginning of the work of the Stain Commission, complaints of basic fuchsin for use in the Endo medium have been common and it has been a mystery to us why some samples have been good and others poor in this technic. Meanwhile the popularity of the Endo medium has been growing less. It is now being largely superseded in various laboratories by other dye media, such as the eosin-methylene-blue agar. This situation is undoubtedly partly because of the other media being intrinsically better; but one cannot help feeling that the Endo medium would be more popular if one could obtain uniformly good results with it, regardless as to what sample of basic fuchsin were employed.

The writers feel that they have at last solved the problem of the Endo medium so far as concerns the varying behavior of different samples of the fuchsin in the medium. It must be explained at the outset, however, that no brief for the Endo medium is offered in preference to other more recently proposed media containing one dye or another that have been shown to be of probably greater value in differentiating organisms of the colon-typhoid group. The writers are merely taking into consideration the fact that the Endo medium is still in wide use among bacteriologists and probably will continue to be for some time yet; this being the case, it is important to standardize either the formula or the fuchsins so that a uniformly satisfactory medium can be made up anywhere.

Like so many other problems which have seemed baffling at first, the solution of this one has proved to be simplicity itself after it has once been learned. It is due primarily to the fact that the ratio of sulfite to fuchsin in the medium is quite important and that this ratio varies with different samples when prepared by the standard formula for the Endo medium—or for that matter, by many of the other formulae for this medium that have occurred in the literature. To explain this matter, one should examine the standard formula of the A.P.H.A., which has been changed slightly from time to time, but has now remained unaltered since 1925. This formula calls for the addition of 100 ml. of the medium to 0.5 ml. of a 10% alcoholic solution of fuchsin, which is then decolorized by the addition of 0.125 g. anhydrous sodium sulfite. Now in practice one can never secure an alcoholic solution containing 10% of basic fuchsin, so the alcoholic solution called for in this formula should actually be saturated, provided sufficient care has been taken to get all the dye possible into

solution. This fact would not cause a serious error if a solution prepared this way always contained the same quantity of fuchsin. Unfortunately, however, it does not. Chemically, a fuchsin may consist of rosanilin or pararosanilin, and either dye may be in the form of a chloride or an acetate; or it may be any mixture of any of these four salts. By referring to the solubility table on page 200 of Biological Stains, 2nd edition, it will be seen that the theoretical solubility of pararosanilin acetate is as high as 13% and of pararosanilin chloride as low as 6%. In practice the theoretically possible amount does go completely into solution, but it is very evident from these figures that the solubility of a sample must vary according to which salt predominates. Another possibility of variation in the strength of the solution comes from the fact that some fuchsin samples dissolve more readily than others, owing to the nature and amount of impurities present. Accordingly, if one has a readily soluble sample he may easily get a saturated solution. With a less readily soluble sample, however, a solution prepared by the same method will not be truly saturated and will contain a smaller amount of actual dye. It is quite difficult for a laboratory worker who has not actually done chemical work on dyes to tell whether or not any more of the sample can be brought into solution.

The outcome of these facts is that one cannot be certain of the actual ratio of sulfite to fuchsin in the standard formula. This ratio is given at the top of Table 1 as about 3:1. It is calculated on the assumption that a saturated solution of fuchsin contains 8% of dye. Inasmuch as the amount actually brought into solution may vary greatly, as just pointed out, the true ratio may sometimes be much greater than 3:1 or sometimes much lower.

There are theoretical reasons for believing that the ratio of sulfite to fuchsin may be quite important in this medium. If, as claimed by Neuberg and Nord (1919), and more recently by Margolena and Hansen (1933), the sulfite acts not only as a decolorizer for the fuchsin but also as a trapping agent for the aldehyde, it should be present in excess of the amount needed to reduce the fuchsin. It is also entirely possible that too great an excess of the sulfite might prevent restoration of the color.

With this thought in mind, the proportion of sulfite to fuchsin in various formulae that have been published for the Endo medium has been studied. There are many different formulae for this medium in existence and upon looking thru the literature one finds that there is a great variation in the amount of fuchsin called for, in the amount of sulfite, and in the ratio between the two, in the various formulae that have been published. To show what variation there has been,

TABLE 1. AMOUNTS OF SULFITE AND FUCHSIN IN VARIOUS FORMULAE FOR ENDO MEDIUM

	100 ml. medium contains			Ratio
		as used	quantity	Sulfite: fuchsin
A.P.H.A., 1925–	Fuchsin Sulfite	0.5ml. 10% alc. sol. 0.125g. (anhyd.)	ca. 0.04g. 0.125g.	ca. 3:1
A.P.H.A., 1920, '23	Fuchsin Sulfite	0.5ml. 10% alc. sol. 0.25g. (anhyd.)	ca. 0.04g. 0.25g.	ca. 6:1
A.P.H.A., 1912, '17	Fuchsin Sulfite	0.1ml. 10% alc. sol. 0.05g. (anhyd.?)	ca. 0.008g. 0.05g. (or 0.025g.)	ca. 6:1 (or 3:1)
S.A.B. Manual	Fuchsin Sulfite	0.5ml. 0.6–1.5% sol. 10ml. 2.5% aq. sol.	0.003–0.0075g. 0.25g.	33:1 to 80:1
U. S. A. Med. Man. No. 6 (1918)	Fuchsin Sulfite	0.18ml. sat. sol. q. s. (e. g., 2.5ml. 10% cryst.)	ca. 0.014g. 0.25g. (more or less)	ca. 18:1
Kendall & Day (1911)	Fuchsin Sulfite	1.0ml. 10% sol. 10ml. 10% aq. sol.	ca. 0.08g. 1g.	ca. 12½:1
Heinemann*	Fuchsin Sulfite	0.5ml. alc. sol (sat?) 2.5ml. 10% aq. sol.	ca. 0.04g. (?) 0.25g.	ca. 6:1
Klinger*	Fuchsin Sulfite	1ml. sat. alc. sol. 5ml. 10% aq. sol.	ca. 0.08g. 0.5g.	ca. 6:1
Kastle & Elvolve (1909)	Fuchsin Sulfite	0.5ml. 10% alc. sol. 2.5ml. 5% aq. sol.	ca. 0.04g. 0.125g.	ca. 3:1
Kinyoun & Deiter (1912)	Fuchsin Sulfite	1ml. half sat. alc. sol. 5ml. 5% aq. sol.	ca. 0.04g. 0.25g.	ca. 6:1
Roddy*	Fuchsin Sulfite	0.3ml. sat. alc. sol. 3ml. 10% aq. sol.	ca. 0.025g. 0.3g.	ca. 12:1
Tanner (1932)	Fuchsin Sulfite	0.5ml. sat. alc. sol. 0.25g. (anhyd.)	ca. 0.04g. 0.25g.	ca. 6:1
Bezançon*	Fuchsin Sulfite	0.25ml. 10% alc. sol. 2.5ml. 10% aq. sol.	ca. 0.02g. 0.25g.	ca. 12½:1
Stitt (1923)	Fuchsin Sulfite	6 drops sat. alc. sol. 18–20 drops 10% aq. sol.	ca. 0.025 ca. 0.1g	ca. 4:1
Proposed formula	Fuchsin Sulfite	1.0ml. 1% alc. sol. 0.125g. (anhyd.)	0.01g. 0.125g.	12½:1

*These formulae are taken from Levine and Schoehlein (1930), the original references not having been located.

a number of representative formulae are compared in Table 1 in respect to the amounts of fuchsin and sodium sulfite called for. This list of Endo formulae is by no means an exhaustive one, but it is long enough to serve for the present purpose.

The first formula in the table is the present standard method of the American Public Health Association, the formula just discussed above. It is next followed by two earlier methods adopted by the A. P. H. A., to show that slight changes have been made in their standard formula from time to time, altho it is hard at present to determine what the reasons for these changes have been. The outstanding characteristic of the present standard formula in comparison with all the others in this table (including the two earlier A.P.H.A. formulae) is, apparently, the very low ratio of sulfite to fuchsin. Only one other formula listed has as a low a ratio, and that one (Kastle and Elvolve) is practically the same as the present A. P. H. A. formula.

The formula in the table immediately following the three taken from the A. P. H. A. publications is the one in Manual of Methods for Pure Culture Study of Bacteria (of the Committee on Bacteriological Technic of the Society of American Bacteriologists) as it appeared in editions previous to 1933. This formula was first proposed by the Committee on Technic because some of the faults of the A.P.H.A. formula were realized, altho the reason for these faults was not known. This formula from the Manual of Methods calls for adjustment of the quantity of fuchsin and, therefore, gives a varying ratio of sulfite to fuchsin. It also avoids the use of a saturated alcoholic stock solution of fuchsin, which is unsatisfactory for the reasons explained above. The ratio in this formula is very much greater than that in the A. P. H. A. medium, running from 33:1 up to 80:1, according to the amount of fuchsin used. This ratio, as will be explained later, proves as much too high as the A. P. H. A. ratio is too low.

The fifth formula given in the table was taken from an early edition (1918) of the U. S. Army Medical Manual, the latest edition (1929) having changed to the A. P. H. A. standard formula. This old formula called for a comparatively small amount of basic fuchsin with an indefinite amount of sodium sulfite, the exact proportion to be determined by a sort of titration. Using the methods of preparing the medium as described in this Manual, it is found that the ratio actually obtained is ordinarily about 18:1. It proves to be a very good formula and it is unfortunate that the latest edition of this Manual has changed to a very much less satisfactory one.

Following this formula in the table are several which are given merely for illustration purposes to show how greatly the quantities

of the two ingredients and the ratio between them vary in different instances. The ratios in these other formulae vary from 3:1 up to 12½:1. The last formula given in the table is the one which the present writers are now proposing. It will be discussed a little later.

Altho the writers have just criticised the standard method of the A. P. H. A., it must be admitted that with some lots of basic fuchsin this formula gives very good results. In making the present comparison between the different formulae and the different samples of fuchsin, good results have been judged by the following criteria: Absence of appreciable color on the plates after cooling but before incubation; dark red growth of *Bacterium coli* (*Escherichia coli*) with a distinct metallic sheen and reddening of the adjacent agar; pink growth of *Bacterium aerogenes* (*Aerobacter aerogenes*) without metallic sheen or reddening of the medium; no reddening of the medium after 48 hours, except in the immediate neighborhood of colonies or streaks of the colon organism. With many samples of basic fuchsin results are not so good. The most common fault in this formula is the one last mentioned—namely, the reddening of the medium, even where there is no growth of the colon organism.

It must be added that one other type of fault in a fuchsin for the Endo medium has been observed in this work. Some samples of fuchsin, upon addition of sulfite, become brown instead of decolorizing to a light pink or straw color. Sometimes this brown color is accompanied by the formation of a dark brown precipitate. This precipitate can ordinarily be brought into solution by the addition of a small quantity of alcohol. Sometimes this also causes the brown color to disappear, but with other samples of basic fuchsin the brown color remains in evidence, even after dissolving the precipitate by the addition of alcohol. In some formulae none of these samples showing the brown color or precipitate could be made to give satisfactory results, but in other formulae they could be used without any difficulty, due to the fact that the brown color becomes inconspicuous after the dye is diluted with the rest of the constituents of the medium. In the A. P. H. A. formula, however, such a sample was almost never satisfactory, due probably to the fact that the stock solution of the dye from which the medium is prepared is saturated and, hence, does not contain any excess alcohol to prevent the formation of precipitate with these dye samples.

The method published in the Manual of Methods for Pure Culture Study of Bacteria shows an entirely different fault. The ratio of sulfite to dye is so high in this formula that one never finds the extreme reddening of the entire medium that is noticed with some samples of fuchsin in the A. P. H. A. formula. The Manual formula,

however, errs in the other direction The excess of sulfite proves to be so great that with some samples of fuchsin not even the colon organism is able to restore the color to any great extent and the characteristic metallic sheen which should be produced by this organism does not appear. As a result, the writers have sometimes found that samples which are poor in the A. P. H. A. formula are good in the Manual formula; but others that are poor in the latter are good in the former.

As soon as it was realized that one of these formulae had too great a ratio for some samples, the other too small a ratio for others, it seemed reasonable to suppose that some intermediate formula might be found which would be usable for almost any sample of fuchsin. Such proved to be the case. The essential particulars of the formula worked out with this idea in mind are given in the last line of Table 1; in greater detail it is as follows:

Dissolve, by boiling 5 g. beef extract, 10 g. peptone, and 30 g. air-dried agar in 1000 ml. distilled water. Adjust reaction to about pH 7.4 and filter thru cotton. Add 10 g. lactose, and allow it to dissolve. Place in small flasks, 100 ml. to each and sterilize for 15 minutes at 15 pounds pressure. Just before use prepare a 1% solution of basic fuchsin (of 85 to 90% actual dye content) in 95% ethyl alcohol. Add 1 ml. of this fuchsin solution to 100 ml. of the agar (melted); also 0.125 g. anhydrous sodium sulfite dissolved in about 5 ml. distilled water. The medium should be light pink while hot and almost colorless after cooling.

Altho the differences between this formula and that in the A. P. H. A. standard method appear very small, they are more important than would be supposed at first glance. The only actual differences are as follows: a 1% instead of a 10% stock solution of fuchsin is specified; of this solution one employs 1.0 instead of 0.5 ml. per 100 ml. of the medium. As a result of these two changes in the formula three very important improvements are brought about: 1) the stock solution of fuchsin is of known instead of unknown strength and varies but very slightly with different samples of the dye; 2) the ratio of sulfite to fuchsin in the finished medium is 12½:1 instead of roughly 3:1; 3) there is an excess of alcohol to prevent the formation of an insoluble precipitate when the fuchsin and sulfite are mixed.

Experiment has proved that this ratio of 12½:1 is about correct. Twenty-two different samples of basic fuchsin, some very pure, some reasonably pure commercial products, and one a very impure commercial sample, have been tried by this technic, and satisfactory Endo plates have been made with all of them. To illustrate the results, Table 2 has been prepared giving the findings with those samples (16 in number) which have been submitted by their manufacturers to the Stain Commission for certification. In this table

the results obtained with these same samples by the S. A. B. Manual formula are given; and in five cases comparative results are also given with the A. P. H. A. standard formula. It will be seen that eight of the samples failed to show sufficient color restoration with the former; while three of the five tested with the latter showed too deep color after incubation. The result of either fault is sure to be insufficient differentiation between the organisms that should be distinguished by the medium. Neither fault occurred with the proposed formula.

TABLE 2. TESTS OF 16 COMMERCIAL FUCHSIN SAMPLES IN THE ENDO MEDIUM

Chemical Nature of Sample	Cert. No.	A.P.H.A. Standard Formula		S. A. B. Manual Formula		Proposed Formula
		Results	Fault of color restoration	Results	Fault of color restoration	Results
Pararosanilin	NF 12	Good		Poor	Too weak	Good
"	NF 15	"		Fair	" "	"
Rosanilin	NF 19	Fair	Too deep	Poor	" "	"
"	NF 24	"	" "	"	" "	"
"	NF 25	Unsatis.	" "	Fair	" "	"
?	NF 7			"	" "	"
Rosanilin	NF 8			Good		"
Pararosanilin	NF 11			Poor	" "	"
"	NF 14			Good		"
?	NF 17			"		"
?	NF 22			"		"
Rosanilin	NF 23			"		"
?	CF 6			Fair		"
?	CF 7			Poor	" "	"
?	CF 11			Good		"
?	CF 13			"		"

The general conclusion from this work is that the solution of the Endo problem may be, not by securing greater reliability of the samples of fuchsin on the market, but rather by altering the standard formula so as to make it suitable to a greater variety of samples of this dye. Two steps to accomplish this have been taken: first, to arrange to have this new formula included in the next revision of the S.A.B. Manual of Methods for Pure Culture Study of Bacteria; and second, to request the A.P.H.A. Committee on Standard Methods of Water Analysis to make a similar change in their next edition.

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NOTES ON TECHNIC

Bismarck Brown for Pollen Grain Walls.—Sections of buds fixed in Bouin's fluid and embedded in paraffin are stained for 6 to 10 minutes with 0.5% aqueous Bismarck brown, rinsed in water, dehydrated in 95% and absolute alcohol. They are then counterstained and differentiated with approximately 0.3% fast green FCF dissolved in clove oil. The sections are passed thru xylol and mounted.

Successful preparations should show a green extine and a brown entine layer of the pollen grain wall. Nuclei are brown, except when in achromatic stage, when they are feebly colored yellowish green with yellow nucleoli. Cytoplasm is green and lignified walls light brown.—Lubow A. Margolena, Geneva, N. Y.

Oil of Cloves for Differentiation in Mallory's Phloxine-Methylene-Blue Stain.—In Mallory's original phloxine-methylene-blue technic, 95% alcohol is used for differentiation after the sections are stained with 5% phloxine, followed by dilute Unna's methylene blue. Wolbach finds that the presence of colophonium in the alcohol is necessary for successful differentiation.

In the writer's experience, clove oil differentiates considerably faster and the pink shows brighter than when alcohol alone, or alcoholic colophonium is used. After the sections (fixed in Zenker's, paraffin embedded) are stained according to Mallory's procedure, they are rinsed in water and dehydrated by dropping upon them 95% alcohol, followed by absolute, after which clove oil is applied. After the pink color has returned to the sections, a microscopic examination is advisable to decide whether the differentiation is complete, or needs continuation. Xylol and balsam follow.—Lubow A. Margolena, Geneva, N. Y.

A Modification of the Dorner Spore Stain.—Dorner's spore stain (LeLait, 6, 8–12, 1926) has the disadvantage of requiring a rather heavy suspension of organisms, and is cumbersome due to the necessity of using test tubes submerged in boiling water. To overcome the above difficulties the following technic was devised under the direction of Dr. James A. Kennedy at Strong Memorial Hospital, Rochester, N. Y.

1) Make a thin film of the organisms on a slide. 2) Cover with a small piece of blotting paper and apply several drops of freshly filtered Ziehl's carbol fuchsin. 3) Allow preparation to steam five to ten minutes on a hot plate, keeping the blotting paper moist with

carbol fuchsin. 4) Decolorize instantaneously with 95% alcohol and wash with water. (This step may be omitted if the spores do not hold the color well, but in general a neater-looking preparation is obtained with its use.) 5) Apply a drop of saturated aqueous nigrosin and spread evenly and thinly over the smear. Allow to dry quickly on the hot plate.

The results obtained (spores red and the vegetative parts of the cells almost colorless) are very similar to those of the unmodified Dorner technic. The procedure is simpler and quicker and is more successful in many cases. The above technic gave good results with spore-formers identified as the following: *Bacillus megatherium*, *B. niger*, *B. cereus*, *B. mycoides*, and with some cultures of *B. subtilis* (Marburg strain). The unmodified Dorner method is quite unsatisfactory for the last named organism; this modified technic is better, but does not give uniformly good results with all strains.—MARION A. SNYDER, Geneva, N. Y.

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stain approved since the last one listed in the January number of this journal.

STAINS CERTIFIED DECEMBER 1, 1933, TO FEBRUARY 28, 1934*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Basic fuchsin	NF 25	78%	For use as bacterial stain, and in Endo medium	Dec. 15, 1933
Hematoxylin	FH 11	——	As histological and cytological stain	Dec. 20, 1933
Malachite green	LMg 1	99%	As histological counterstain	Jan. 4, 1934
Carmin	NCa 4	——	As histological and cytological stain	Jan. 15, 1934
Pyronin G	NP 4	32%	As histological and bacteriological stain	Jan. 23, 1934
Crystal violet	NC 7	90%	As histological, cytological, and bacteriological stain	Feb. 7, 1934

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

PRESENT MEMBERS OF THE COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

The latest published list of the Commission members appeared in January, 1929. There has been considerable change in the membership and in the addresses of the old members who are still on the Commission. The following is an up-to-date list:

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E. G. Hood, Dairy & Cold Storage Branch, Ottawa, Canada.
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Walter G. Sackett, Colo. Agric. College, Ft. Collins, Colo.
S. A. Scudder, City Hospital, Welfare Island, New York, N. Y.
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Conway Zirkle, Dept. of Botany, Univ. of Pennsylvania, Philadelphia, Pa.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked.

BALDWIN, J. T. A method for concentrating and fixing free-living protozoa on cover glasses. Science, **79**, 143. 1934.

The difficulty in obtaining a large number of protozoa on one cover glass is greatly overcome by the following method. Using mimeograph paper (or some equally porous type) make a box about 20 mm. deep,—of a size into which the cover glass will fit closely. Smear the glass thinly with egg albumen and place it face up in the box. Stand the box on blotting paper and fill it with fixative to a depth of 4 mm., or more if the cultures are poorly populated. Into the fixative pipette an equal, or less, amount of water containing the protozoa. The fluid seeps out and is absorbed by the blotting paper. Because the seepage is relatively slow, the organisms are left securely fastened and evenly distributed on the cover glass. When only a thin film of the fluid still envelops the organisms, remove the cover glass and drop it face down into a dish of the fixative. After fixation follow the usual procedure. Reverse the procedure for rhizopods, e.g., transfer them first, and add the fixative after they have become attached.

Various fixing solutions and staining methods give good results by this method, but Navaschin's fluid is particularly recommended for sticking the organisms to the glass. The crystal-violet-iodine stain is excellent for studying nuclear divisions in total mounts of protozoa. The nuclear behavior in ex-conjugants of *Paramacium caudatum* was easily followed by using this method, since the chromosomes are especially sharply defined.

BERCOVITZ, Z. Modified technic for making Wright's blood stain. J. Lab. & Clin. Med., **19**, 438. 1934.

The following modification of Wright's stain is recommended as it avoids precipitates and is more economical than the usual procedure. Prepare blood films and air dry. Immerse in jar with methyl alcohol for 5 or more minutes. Transfer to Wright's stain prepared by dissolving 1 tabloid (Burroughs Wellcome Co.) in 10 cc. pure methyl alcohol, diluted with distilled water up to 30%. Stain for 2 to 3 minutes, depending on the intensity desired, and wash. The stain solution can be used repeatedly.

CARTER, J. S. Reactions of *Stenostomum* to vital staining. J. Exp. Zoology, **65**, 159–179. 1933.

A number of dyes were used for the vital staining of three species of *Stenostomum*, especially *S. grande*. The animals were placed in a ring of vaseline on a slide with a very thin cover glass applied, and studied under water immersion. Alizarin (Kepler and Carter) or rongalit (McConnell) render the internal structure of the animal more apparent without the staining. One drop of a 0.001% aqueous stain was added to 5 cc. of the medium; in the case of alcoholic solutions, only the amount that would adhere to the end of a needle was added to the same amount of the medium. *Stenostoma* was sectioned after successive days in the progress of staining. Helly's fluid did not alter the color of rhabdites when stained in toluidine blue or in Bismarck brown.

A small amount of the vital dyes accumulated in the vacuoles, but a far greater quantity accumulated in the rhabdites. A table is given telling how the various colors appear in rhabdites of different layers. In a general way, all the stains encounter an acid medium in the rhabdites. In their progress in the animal body, they encounter a more and more alkaline medium. Benzene-azo-alpha-naphthal-amine (called BAN) colored intensely and rapidly, and could be followed thru the body more easily than any of the other stains. The rhabdites in the epidermis were red, orange in the mesenchyme, and yellow in the enteron. Bismarck brown stained rhabdites in the epidermis orange-red, yellow-orange in the mesenchyme, and yellow in the enteron. Unlike the other stains, it caused the rhabdites to break up into spherules before the epidermal cells were removed from the epithelium.

Toluidine blue has no striking effects, but proved least toxic and the animals, therefore, could be left in it for considerable time. Brilliant cresyl blue and Nile blue sulfate resemble toluidine blue in their action. Janus green, in very dilute solution, stains rhabdites an orchid shade, and blue in stronger concentration. The rhabdites turn pink in the mesenchyme and blue in the enteron. Neutral red stained rhabdites a dark red in a very weak concentration, and the internal structures could not be distinguished. Trypan blue failed to stain. Methyl red stained similarly to BAN. Alizarin, 1 drop to 5 cc. of the medium (1 cc. alcoholic alizarin to 50 cc. of water) remained yellow in the rhabdites, but much deeper in the enteron. It caused accumulation of dark blue concrements in the distended anterior end of the main stem of nephridial tubules. Wright's stain colored only the rhabdites. Eosin-azure II did not stain directly, but appeared blue after entering the mesenchyme (being carried by unstained epidermal rhabdites). Rongalit gives a similar reaction.

Of the 3 acid dyes used, 2 were trapped by rhabdites, and the third failed to accumulate. Interesting physiological effects in connection with the staining are described. The experiments prove that staining does not inhibit growth, fission or gonad formation.

CLARA, M. Über ein neues Molybdänhämatoxylin. Ztz. Wis. Mikr., **50**, 73–77. 1933.

The stain recommended by the author may be a connective tissue, differential, or nuclear stain, depending on the reaction of the water used after its application. To a 1% aqueous solution of hematoxylin add an equal quantity of a 10% solution of ammonium molybdate. Add molybdic acid to saturation. The solution appears bluish violet when fresh and becomes dark red after standing. As with Held's method, this solution should be diluted with distilled water for use. The sections need no mordanting and are transferred to the hematoxylin from distilled water.

Stain for 24 or more hours, and rinse in distilled water. If overstained differentiate in picric acid, and rinse in 80 and 90% alcohol. Results: Connective tissue wine-red to brown-red, other tissue unstained or a weak reddish hue. If the sections are left in the distilled water for several hours, the connective tissue stain fades out, while the nuclei begin to show some coloration. If tap water is used instead of distilled, and especially if a little (only traces) of lithium carbonate is added to the tap water, the picture changes; as the connective tissue becomes colorless, while the nuclei appear blue and sharply defined.

GAY, F. P. and CLARK, ADA R. The differentiation of living from dead bacteria by staining reactions. J. Bact., **27**, 175–89. 1934.

An investigation has been made of the Proca-Kayser stain for differentiating living from dead bacteria. In this technic a bacterial smear which has been fixed in the ordinary manner by drying and flaming is stained for 3 to 5 minutes in Loeffler's alkaline methylene blue, followed by rapid washing and staining for 5 to 10 seconds only in Ziehl-Neelsen's carbol fuchsin with subsequent washing. If the time indicated is followed carefully in each case, the method is supposed to stain living bacteria blue and dead bacteria purple to red. The writers' investigations indicate that the method is quite accurate and that apparent exceptions are found on careful study to be actually confirmatory of the accuracy of the method. Spores, however, do not behave like the vegetative cells. Living spores stain very faintly, if at all, while the dead spores stain blue.

The writers find it possible to replace the carbol fuchsin in this technic by aqueous basic fuchsin or safranin or, with less satisfactory results, by Bismarck brown or alcoholic eosin. The Loeffler's methylene blue may be replaced by saturated aqueous methylene blue or, with less satisfactory results, by toluidine blue or thionin. (The sources and exact chemical nature of the dyes are not stated.)

The effect of neutral red on bacteria before and after ingestion by phagocytes is investigated, and it is found that the dye does not stain the bacteria until they have been ingested. It is concluded that this reaction indicates injury to the cell rather than simple death.

GRAUBNER, E. Etude de l'influence des fixateurs sur le glycogène du cartilage. Bull. d'Histol. Appl. **10**, 214–223. 1933.

The author tested a number of described methods for the fixing and staining of glycogen in sections. His studies were made on thyroid cartilage of cows, horses, pigs

and sheep He concludes that formol, aqueous sublimate, Zenker's fluid, and Müller's formol are suitable fixatives. Alcoholic solutions and formol saturated with glucose are detrimental. Frozen sections are preferred to embedded ones. Best's staining is recommended (Best, Zts. Wis. Mikr., **23,** 319, 1906), but it should be prolonged to 30 or 45 minutes.

HÄGGQVIST, G. Eisenchloridhämatoxylin. Zts. Wis. Mikr., **50,** 77–82. 1933.

The author has used the oxydizing and mordanting quality of ferric salts for hematoxylin. Ferric chloride has proved to be excellent for hematoxylin staining. For zoological material, fixatives containing sublimate, e. g., Carnoy's and trichloracetic acid, are recommended. A 20% solution of formalin saturated with $HgCl_2$ at room temperature (not over a month old) has been used for 15 years in the author's laboratory and is preferred to other fixatives.

Remove $HgCl_2$ from sections with iodine dissolved in 70% alcohol, follow with a 0.25% solution of sodium thiosulfate to remove the iodine. Rinse in distilled water and mordant in ferric chloride. For the progressive method, use a 3 to 5% solution for 5 to 30 minutes, rinse and stain in freshly prepared hematoxylin. For the regressive method, use a 5% solution of ferric chloride for 1 hour. Stain in 1% fresh hematoxylin for at least 1 hour. Rinse and differentiate in 1 to 3% ferric chloride. The 1% solution is preferred because the details are better brought out if destaining is not too rapid.

The results are quite similar to those obtained with Heidenhain's hematoxylin, except that the staining is blue and not black, as in this method fresh, unoxydized hematoxylin is used. Differentiation seems easier than with iron alum.

The procedure is very well suited for muscle, gland and various cell structures in general.

KAMPMEIER, O. F. Some simple methods of preparing and mounting cross sections of the body, of illustrating microscopic sections, and of labeling specimens for the anatomical museum. Anat. Rec. **55,** 395–401. 1933.

By the time the human body reaches the museum it has to be preserved and artificially colored. Embalm the selected body with 10% formalin, freeze and cut sections with a band saw about 1 inch thick. After thawing, clean the sections, soak in several changes of water, and store in 5% neutral formalin, until further need. Fasten loose parts, such as the loops of intestine with gelatin. Drain excess fluid off and lay the sections on oiled paper. Pour warm liquid gelatin into crevices until it oozes out of the margins of the section, add a quantity sufficient to unite all loose structures upon solidification.

Pipette hot gelatin colored with neutral carmin on muscles. Stain the fat in subcutaneous areas and elsewhere by brushing on hot gelatin colored yellow with a fine suspension of lead picrate, (prepared by adding picric acid to lead acetate and washing off the fine insoluble precipitate with distilled water).

After the blood coagula have been removed, and the lumen of the vessels have been stuffed with cotton, drip melted wax of the following composition: 1 pt. paraffin, 2 pts. pure beeswax and vermillion or cobalt blue (artist's oil colors) upon the remainder of the lumen. Use vermillion for arteries and cobalt blue for veins. For smaller vessels use glass beads of proper size and color. Indicate the ducts in a similar way.

For mounting use strips of non-corrosive monel metal, 1.5 mm. thick and 2.2 cm. wide. Bend the strips so as to conform to the outlines of the lower half of the section, using the ends of the strips for legs, adapted to fit snuggly against the inner sides of the jar, forming a supporting hammock for the preparation. Tie the section by a few loops of silk thread to the strip, and place in jars. Preserve in neutral 5% formalin, depositing white marble chips at the bottom to prevent acidity. A method for the preparations of wax models and for labeling museum specimen is also given.

PETRUNKEVITCH, A. New fixing fluids for general purposes. Science, **77,** 117–8 1933.

The fluids recommended here are the result of many years of experimentation and are more or less free of the usual defects of hardening of the tissues or of interference with staining. Animals fixed in them remain soft and do not harden subsequently in 70% alcohol, in which they may remain for many weeks. All common stains may be used. Mallory's triple stain gives brilliant differentiation, tho the picture is somewhat different from that following Zenker's fluid.

Phenol has been found to give a peculiar elastic texture to the tissues. Paranitrophenol comes nearest to phenol in this respect and, being stable in mixture, is preferable where stability is desired. The author used both for arachnids, insects, myriapods, leeches, earthworms, flatworms, roundworms, older amphibian larvae and mammalian embryos. Macroscopic dissection of invertebrates and mammalian embryos fixed in either of the fluids is greatly facilitated.

Cupric-phenol fixing fluid. Stock solution A: Distilled water, 100 cc.; nitric acid, (c.p., sp. gr. 1.41–1.42) 12 cc.; $Cu(NO_3)_2 \cdot 3H_2O$, 8 g. Stock solution B: 80% alcohol, 100 cc.; phenol crystals, c.p., 4 g.; ether, 6 cc. The stock solutions are stable. For use take 1 part solution A and 3 parts solution B. The mixture does not keep and must be used within a few hours. For the same reason, the duration of fixation must not exceed 48 hours, 12 to 24 hours usually being sufficient. Wash in 70% alcohol.

The following fluids are perfectly stable and may be kept in glass-stoppered bottles for months.

Cupric-paranitrophenol fixing fluid: 60% alcohol, 100 cc.; nitric acid (as above), 3 cc.; ether, 5 cc.; cupric nitrate (as above), 2 g.; paranitrophenol, c.p., crystals, 5 g. Duration of fixation not limited by time, except as to the minimum time required for penetration at the rate of 0.5 mm. per hour. Wash in 70% alcohol.

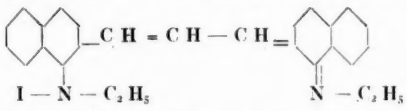
Fixing fluids may be used of the same composition as the latter except that in place of 5 g. of paranitrophenol they contain 0.5 g. of one of the following derivatives of phenol: orthonitrophenol; alpha (2:4) dinitrophenol; beta (2:6) dinitrophenol; picric acid (2:4:6 trinitrophenol). Fixation and washing as with paranitrophenol, but the last two require longer washing and leave the tissues yellow.

POLICARD, A. Les matières minérales fixes des éléments séminaux au cours de la spermatogénèse. Bull. d'Histol. Appl., **10**, 313–20. 1933

The author finds that micro-incineration reveals some very fine yellowish ash (due to iron) in the anterior portion of the head of spermatozoa and abundant white shiny ash in the posterior. The middle portion and the tail show very little mineral matter. The evolution of the mineral matter was studied during spermatogenesis in the testis of rats, (alcohol-formol fixation, paraffin embedded, etc., followed by micro-incineration). Ash was very considerable during the spermatogonial and spermatocyte stages, while spermatids showed very little mineral matter. A progressive increase to the spermatozoa stage follows. Sertoli's cells show very little ash, but residual bodies a considerable quantity.

PROESCHER, F. Pinacyanol as a histological stain. Proc. Soc. Exp. Biol. & Med., **31**, 79–81. 1933.

Pinacyanol is a dye belonging to the cyanine group. Its formula is



and it may be obtained from the Eastman Kodak Co., Rochester, N. Y. Pinacyanol is recommended for morphologic and chemo-histologic staining of frozen sections. Formol and formol-Müller's fixatives are recommended. Paraffin or celloidin sections do not show the brilliancy of details obtained with frozen sections. For staining prepare a 0.1 to 0.5% solution in absolute ethyl or methyl alcohol. In glass stoppered bottles, protected from light, the solution keeps indefinitely. Flood mounted sections for 5 to 10 seconds, transfer to distilled water for about 15 seconds, remount and embed in glycerin.

Results: Chromatin dark blue to cobalt violet, protoplasm various shades of purple, connective tissue eosin-red, elastic tissue deep black-violet, and muscle from bluish violet to purple. Neutrophilic and eosinophilic granules remain unstained, while the granulo-plasma of the plasma cells is a brilliant carmin red. Hemoglobin remains unstained; neutral fat is colorless or of a faint bluish violet hue. Various lipoids are deep bluish violet to deep purple. Amyloid distinctly carmin red. Hemosiderin is a brilliant orange-yellow. Glycerin seems to intensify the staining.

REYNIERS, J. A. Studies in micrurgical technique. V. A moist chamber for tissue culture and cellular dissection Anat. Rec., **56**, 295–305. 1933.

The moist chamber whose construction is described, involves a new application of the cross slide, floating center principles. The chamber may be kept closed during work in which the experiment is carried over long periods of time. This allows for a minimum of change in the physical conditions within the chamber. Gaseous pressures may be built up and maintained at will. The chamber may be heated to any desired temperature and will maintain itself automatically at this temperature. This apparatus can be used with most micro-manipulators now in use or it can be worked by hand. It can be constructed larger or smaller to be worked under high powers or beneath the lower powers of a dissecting microscope, the sweep of the pipette being adequate under any power. The pipette is moved in an arc movement, but a moist chamber has been constructed on this principle so that the pipette tip is moved in straight lines. Specimens may be worked on the under side of a cover slip or they may be worked on the floor of the chamber if it is built slightly lower.

REYNIERS, J. A. Studies in micrurgical technique. VI. A micro suction and injection apparatus. Anat. Rec , **56**, 307–13. 1933.

A micro suction and injection apparatus is presented. This apparatus may be built from laboratory odds and ends. The principle employed is the compression of an air column by a regulated stream of mercury. The air compression can be built up as slowly as the operator wishes and may be instantly returned to normal by a check valve, thus effecting an immediate and absolute control of the suction or injection. Since pushers of mercury, water, and the like are done away with in this apparatus, there is little danger of contaminating or diluting the injected fluid or injuring the cell.

SHIBATA, Y. Vergleichende Beobachtung mit der Fixierungsweise der verschiedenen Fixierungsmittel, besonders unter Berücksichtigung ihrer Wasserstoffionenkonzentration an den Nissl-Körperchen der Ganglienzellen. Folia Anatomica Japonica, **11**, 275–84. 1933.

The appearance of Nissl bodies, when stained with toluidine blue, depends not only upon the physiologic condition of the ganglionic cells, but also upon the fixatives, and especially upon the reaction of the latter. Spinal cord of the rabbit and dog served for material. The fixatives used were: Hamilton's, Orth's, absolute alcohol, Zenker's, 10% formol, and Rath's.

Tissue after fixatives containing potassium bichromate show a diffuse blue staining of the plasma, or irregular granulations and stained nuclear granules. Luna's and Müller's fixatives hinder the staining, while alcohol, formol, and sublimate bring out Nissl bodies and gliar nuclei clearly. When 10% formalin is used with phosphate mixtures (buffers) the pH-value remains constant before and after fixation. The reaction of the fixatives influence the staining in the following manner: the more alkaline the fixing fluid the more intense the color when basic stains are used. The most suitable reaction for fixing Nissl bodies is around pH 5.3.

SLIFER, E. H. and KING, R. L. Grasshopper eggs and the paraffin method. Science, **78**, 366. 1933.

Petrunkevitch's fixative No. 1 (Science, **77**, 117, 1933) was employed on the eggs, testis and young embryo of grasshoppers. The eggs, which had hitherto proved extremely refractory in the paraffin technic, were easily sectioned following this fixative. For a cytological study of the testis and embryo, however, the fixative proved quite unsatisfactory; cytoplasmic details were distorted, and chromosomes were almost unrecognizable.

The authors then tried to use No. 1, or parts of No. 1 after Bouin's solution. The following procedure is recommended for grasshopper eggs: Fix in Carnoy-Lebrun's, as suggested by McNabb (J. W. McNabb, J. Morph. & Physiol., **45**, 47, 1928). Wash in iodized alcohol, cut in half and store the micropyle halves in 70 to 80% alcohol until needed. Treat with 4% phenol in 80% alcohol for 24 hours, dehydrate in 95%, clear in carbol xylol, infiltrate and cut with the cut end out. Trim the paraffin away until the yolk is just exposed and soak in water for 24 to 48 hours. As an alternative method, after the material has been dehydrated in 95% alcohol, it may be cleared in anilin oil, washed in chloroform, embedded, and then placed in water as above. Preparations of

various stages of the eggs of *Melanophus differentialis* and *Chortophaga viridifasciata* have been successfully obtained by this method. Fuelgen's reaction, since it does not color the yolk, has been found an important aid in the study of such material.

The essential features of this process are the application of phenol and soaking in water. It is not unlikely that the method will prove useful for other objects that are difficult to section.

THIBAUDEAU, A. A. The preparation and staining of frozen sections. J. Lab. & Clin. Med., **19**, 204–9. 1933

The procedure described is the result of 20 years of experience with various methods at the Pathological Laboratory of the N. Y. Institute for the Study of Malignant Disease, Buffalo, N. Y. The whole process of sectioning, mounting and staining may be carried out in 5 minutes and gives excellent and permanent preparations.

The microtome recommended is a rigid, large sledge instrument (Spencer No. 860) modified for a freezing chamber, used with 18 cm. or 25 cm. knives. Ten per cent formalin is preferably used for fixation. Carefully selected blocks, including if possible pathologic and normal portions, are cut 1 to 2 cm. square by 2 to 3 mm. thick. Freezing is accomplished with CO_2 by short blasts to the desired consistency. Water is used for embedding the material. The block is trimmed while being frozen. Best sections are secured when the freshly stropped knife is inclined at about 25° to the horizontal and sweeps over the block at an angle of approximately 30°. Remove sections to 10% alcohol and stain in Harris' hematoxylin for 5 to 15 seconds. Rinse in distilled water until all excess color is removed. Transfer to distilled water with a few drops of NH_4OH to change the color to blue. Pass thru 70, 85, and 95% alcohol, respectively. Counterstain in saturated eosin in absolute alcohol for 5 seconds. Clear in carbol xylol and mount directly on slide without section lifter. Blot with filter paper and mount in balsam.

ZWEIBAUM, J. Sur un nouveau procédé de coloration des graisses. Bull. d'Histol. Appl., **10**, 210–3. 1933.

Of the various fat stains Sudan III and osmic acid are most frequently used. Osmic acid, however, also blackens bodies other than fat, while Sudan III fails to stain palmitines and stearines. The author discusses fixatives and mentions that formalin causes fusion of the fat droplets, which eventually decolorize. Zenker's fixative is preferred, but the color after its application is not permanent. Szantroch's method gives similar results.

Osmic acid deposits a film around the fat droplets, which inhibits their fusion, and the action of osmic acid is, therefore, to be recommended. The following solution has been successfully used for cells cultivated in vitro and for frozen sections: 1% osmic acid, 2 parts; 1% chromic acid, 3 parts; 3% potassium bichromate, 3 parts. Fix 3 to 24 hours, wash in tap water 10 to 15 minutes or longer, depending on the time of fixation, transfer to 50% alcohol, and stain in Sudan III in 70% alcohol for 5 to 10 minutes. Wash and stain in dilute Delafield's hematoxylin, or with thionin. (The latter is not permanent.) Wash and mount in Apathy's syrup.

The smallest fat droplets stand out scarlet or brown-red on a colorless cytoplasm. No fusion takes place and the preparations are permanent. Pieces of tissue require 24 hours fixation, which renders them brittle if they contain much fat. In this case use 20% formol for 24 hours, wash and cut frozen sections 10 mμ thick. Transfer these sections to the chromosmic mixture for 15 to 30 minutes, and proceed as in the first case.